Characterization of Fluoride Ion Channel interactions with synthetic crystallization chaperones

Master’s Thesis

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Christopher Miller, Principal Investigator and Advisor
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ACKNOWLEDGEMENTS

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ABSTRACT

Characterization of Fluoride Ion Channel interactions with synthetic crystallization chaperones

A thesis presented to the Department of Biology

Graduate School of Arts and Sciences
Brandeis University
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By Abraham Z. Cheloff

The Fluc ion channel family comprises dimeric membrane proteins functioning to expel excess fluoride (F\textsuperscript{-}) from the cytoplasm of microorganisms to resist the inhibitory effects of this environmental xenobiotic anion. Recent structures of the \textit{E. coli} Fluc homolog, EC2, bound to engineered “monobody” proteins selected from phage display libraries reveal multiple side-chain contacts at the channel-monobody interface. Two such monobodies, S9 and S12, share a similar interface structure, but their nanomolar-range binding affinities differ by \(~15\) fold. We focus on the per-residue energetic contributions to the binding affinity of the S12-Fluc complex by introducing point mutations at polar contacts located at the interface, assessing the change in binding affinity using fluorescence anisotropy. Analysis of these results in comparison to previous work on S9 indicates that while the residues contacting the monobody-EC2 interface are similar for both S9 and S12, the terminal hydroxyl of various tyrosine residues appear crucial to the binding of the S12-Fluc complex, while relatively insignificant to S9-Fluc.
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Introduction

Properties of Fluoride

A ubiquitous, environmental anion, fluoride is found in both soil and water, forming the 13th most plentiful element in the Earth’s crust[1]. Aqueous fluoride is typically found in the environment at levels that range from 10-100 μM[2] further supplemented with 50-100μM public fluorination, a level high enough to inhibit cellular functions[3]. Although the epithelial cells of animals and other larger organisms provide protection against ubiquitous fluoride, the plasma membranes of bacteria, yeasts, protozoans, and other unicellular or small multicellular organisms provide no such protection[4]. Since hydrogen fluoride is a weak acid, with a pK_a of 3.4, and is membrane permeable, it is susceptible to the “ion trapping” effect. At moderately acidic extracellular conditions, fluoride ions become protonated, and hydrogen fluoride, a small non-polar molecule, is able to diffuse across plasma membranes. Once inside the cell, and at physiological pH, hydrogen fluoride dissociates into its components, leaving fluoride anions trapped in the cell due to its negative charge (Figure 1)[4].

Figure 1: Mechanism of the “ion trapping” effect relevant to hydrogen fluoride. The effect is further aggravated by low extracellular pH levels. At the levels noted above, fluoride would accumulate into the cell in the millimolar range[4].
Due to the ion trapping effect, concentrations of fluoride anions in cells can be higher than that of the environment, rising to the millimolar range. Fluoride ions at these concentrations inhibit key phosphoryl-transfer enzymes needed for energy production and nucleic acid synthesis[1], leading to toxicity and cell death[4].

**Fluoride Ion Channels**

In order to protect themselves from these cytotoxic effects, microorganisms have evolved membrane channels to expel intracellular fluoride[3]. Two such independently evolved proteins are the CLC\(^F\) – type F\(^-\) / H\(^+\) antiporter, a subtype of the CLC class of anion-transporter proteins, and the group of small, constitutively open membrane channels known as crcB or ‘Fluc’[1, 5]. The Fluc family of proteins are found across a wide range of prokaryotic and some eukaryotic organisms and is encoded by the crcB gene[6]. Controlled by a fluoride activated riboswitch, expression of the Fluc channel is dependent on sufficient levels of intracellular fluoride[6], providing a positive regulation mechanism to control both when and how much Fluc is expressed. The *E. coli* Fluc homolog (EC2), which is the focus on this project, is exceptionally specific for fluoride, fluxing ~10,000,000 ions/s, while being impermeable to Cl\(^-\), a relatively abundant environmental competitor[1]. This channel has a dual-topology architecture that is novel for ion channels, a dimer with subunits arranged in an antiparallel fashion[5]. While this structure has not been seen in ion channels before, it has been observed in the SMR family of multidrug transporters, suggesting that the Fluc proteins could be evolutionarily descended from or related to the SMR family of transporters[1].
Monobody Proteins

In confirming the topology and extrapolating the structure of Fluc, engineered binding proteins termed “monobodies’ were developed[5]. These proteins, which are ~10 kDa, are derived from the 10th fibronectin type III domain of human fibronectin. Using phage-display libraries, the sequences were diversified and selected to yield water soluble proteins that fold stably, are cysteine-free, and bind to their targets, such as EC2, selectively with nanomolar affinity[5]. A sample of the results are shown in Figure 2. S9 and S12, the two monobodies investigated here, were each selected from a “Side” library. This library is designed to introduce diversity in both the FG loop and residues along the concave beta strands of the monobody scaffold.

![Monobody Phage-Display Libraries](image)

**Figure 2: A selection of “Side” library phage display results.** The monobody proteins S9 and S12 share remarkably similar sequences, save for the diversified FG loop region[5].
Monobody-Fluc Complex Crystal Structure
As Fluc ion channels are always in an open state, these monobody proteins, which bind to and block Fluc mediated F⁻ current[5], can help us to understand the structure and mechanisms of these channels. Using single channel recordings of Fluc in lipid bilayers, it was shown that various monobody proteins designed to bind to Fluc were able to block the flow of fluoride when exposed to either side of the membrane[7]. This led researchers to conclude that the same binding epitope is exposed on each side of the membrane, and thus supports the notion of an antiparallel dimer topology[5, 8]. Furthermore, crystal structures of EC2 and other Fluc homologs were obtained via the use of monobody proteins, as Fluc protein crystallization is mediated via monobody contacts, forming a lattice of EC2 and monobody proteins[9]. The crystal structure of EC2 bound to S9 shows monobody proteins bound to either side of the channel in a symmetrical fashion (Figure 3). Additionally, the channel is composed of two identical dimers arranged in antiparallel fashion. Each dimer consists of 4 transmembrane alpha helices, consistent with the structure of small multidrug resistance (SMR) channels that Fluc is believed to be related [10].

![Figure 3: EC2 Fluc ion channel homodimer (green and turquoise ribbons) bound to two S9 monobody proteins (magenta).](image)

Relevant Monobody Structure and Sequence
The structural similarity of the different monobody proteins, which each have distinct binding affinities, has led to some curiosity regarding the contributions of individual amino acids to the
binding affinity of these monobodies. In particular, the structures of S9 and S12 are remarkably similar, showing an overall backbone carbon structural alignment within an angstrom (Figure 4a). Their amino acid sequences again show great similarity, with most diversity occurring within the previously defined FG loop region (Figure 4b).

![Figure 4](image)

**Figure 4: Structural and sequential comparison of S9 and S12.** S9 is shown in purple, and S12 is shown in yellow. (a) Structural overlay of S9 and S12 structures determined through x-ray crystallography when bound to EC2. RMSD = 0.70 angstroms. (b) Amino acid comparison of S9 and S12. Bolded residues are distinct between the two structures.
**Energetic Contributions to Monobody Binding Affinity**

While S9 and S12 share similar structures and sequences, their binding affinities to EC2 are quite different. While S9 binds with a $K_d$ of $210 \pm 70\text{nM}$, S12 binds to EC2 with a $K_d$ of $10 \pm 6\text{nM}$. The variety of amino acid residues present in the FG loop region between the S9 and S12 monobodies suggest that there are both electrostatic and volumetric factors leading to such a difference in binding affinity between these two proteins. Elucidating the exact contribution of each amino acid will not only lead to a greater understanding of the biochemical mechanism of binding for these proteins but can hopefully be applied to future work in protein engineering. Receptor binding affinity is an important consideration in most if not all communication pathways between cells, and so applications for this research are widespread.

**Previous Work**

Single channel recordings of EC2 activity with and without monobody present have been used to further understand the kinetics of monobody binding, and thereby accurately measure dissociation constants. Comparison of wildtype S9 and S12 dissociation constants measured with single channel recordings with their fluorescence anisotropy counterparts show great agreement, and thus validates anisotropy as our assay. [11]

Key residues on the S9 monobody have been previously mutated in order to understand the importance of each residue, and conclude how each contributes to binding affinity [11, 12]. Mutations were classified as “severe” if $\Delta \Delta G^o$ was greater than the upper limit of the assay (3.4 kcal/mol), “moderate” for a $\Delta \Delta G^o$ 0.6-3.4 kcal/mol, and “indifferent for a $\Delta \Delta G^o <0.6 \text{ kcal/mol}$, as thermal energy at room temperature is 0.6 kcal/mol. This work revealed an inability to increase binding affinity beyond that of wildtype, suggesting that the interplay of amino acids leading to
the 15x difference in affinity between S9 and S12 may be more complex than initially suspected. Furthermore, mutagenesis revealed some surprises in comparison to the expected results given the crystal structures. Multiple tyrosine residues in the FG loop region were changed to phenylalanine in order to remove any hydrogen bond donor ability. Y78, a residue without a nearby hydrogen bond acceptor, showed a moderate change in affinity when mutated, while Y80 and Y84 were classified as indifferent, even with nearby acceptors on EC2. These results showed a clear need to look beyond supposed electrostatic interactions, and to more critically investigate the spatial or lipophilic energy contributed by each residue.
Materials and Methods

Reagents
Reagents were purchased from Sigma Aldrich or Fischer Scientific at the highest quality available. n-decyl-maltoside (DM) was purchased from Anatrace. EDANS C2 maleimide was purchased from Molecular Probes.

Selection of Residues
The crystal structure of EC2 in complex with S12 monobody was uploaded to PISA (Proteins, Interfaces, Structures and Assemblies). Interfaces were analyzed and supposed Hydrogen-bond or salt bridge interactions between EC2 and S12 were manually investigated in PyMOL. Any residues showing a polar intermolecular interaction with a bond length of less than 4 Å was considered a possible electrostatic interaction. Every amino acid in the diversified FG loop region showing interface contact capability was also individually mutated. Any polar intermolecular interactions with a bond length less than 4 Å was considered a possible electrostatic interaction.

Mutagenesis and Purification of DNA sequences
DNA oligonucleotides for Quickchange™ Site Directed Mutagenesis were designed using Lasergene SeqBuilder Pro and met all primer design specifications laid out in the Quickchange™ Site Directed Mutagenesis instruction manual. Primers used for our mutagenesis were ordered from Integrated DNA Technologies (IDT), and can be found in Supplemental Table S1. The S12 gene with a non-perturbing A12C mutation for conjugation, connected to an N-terminal His6 tag with a short linker containing a TEV protease cut site all in a pHFT1 vector was used as DNA template in a Site Directed Mutagenesis PCR reaction that met instruction manual protocols,
followed by a DpnI digest for 1 hour at 37°C. Alternatively, overlap extension PCR cloning was used[13], followed by ligation into a fresh vector. 1μL of the final reaction mixture was transformed into XL-1 Blue Supercompetent cells, plated on LB containing kanamycin, and left overnight at 37°C. Single colonies were selected, and plasmids were extracted via the QIAspin Spin Miniprep Kit. Plasmid sequencing was completed via Genewiz, using T7 and T7-term primers, to confirm the presence of the desired mutation.

**Expression, Labeling, and Purification of Monobody**

Mutant S12 plasmid was transformed into *E. coli* BL21-(DE3) cells and plated on LB containing kanamycin overnight. All colonies were scrapped into 1L aliquots of terrific broth supplemented with 50 μg/mL kanamycin. Cultures were grown at 37°C and shaken at 220 rpm until an A600 of 0.6-0.8 was reached, and then induced with 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 30°C. Cells were centrifuged at 4000rpm for 18 minutes and stored overnight at 4°C. The bacterial pellet was suspended in 100mM NaCl and 50mM Tris-HCl (pH7.5) and supplemented with 1 μg/mL leupeptin, 5mM TCEP, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were sonicated for 30 seconds, and rested for 30 seconds, for a total time of 6 minutes. Supernatant was clarified via centrifugation at 8000 rpm for 50 minutes. Clarified supernatant was loaded onto a cobalt-affinity column pre-equilibrated with 100mM NaCl and 50mM Tris-HCl (pH7.5), and further washed until flowthrough A280<0.05. TEV protease containing a His6 tag was added (0.2mg/L bacteria) and left for 16 hours at room temperature. Cleaved monobodies were eluted in 100mM NaCl and 50mM Tris-HCl (pH7.5) and concentrated to 10mg/mL. Monobodies were fluorophore labeled with two sequential additions of 1 mM EDANS C2 maleimide 30 minutes apart, each following a room temperature incubation, in the dark. The reaction was quenched with 1mM L-cysteine. The reaction mixture was placed on a desalting column equilibrated with 100mM NaCl and 50mM Tris-HCl (pH7.5), Additional buffer
was added until the total volume placed on the column was 2.5mL, and then the labeled monobody was eluted with 3.5mL 100mM NaCl and 50mM Tris-HCl (pH 7.5), and again concentrated. Final purification occurred on a Superdex S-75 size exclusion column in 100 mM NaCl, 10mM HEPES-NaOH, and 10mM NaF (pH 7). Purified monobodies were stored at concentrations of 50-150 μM at −20 °C.

Expression and Purification of EC2
The EC2 gene containing a yield-enhancing, non-perturbing R25K mutation, with a C-terminal His<sub>6</sub> tag and short linker in a pET-21a(+) vector (termed wildtype) was transformed into E. coli BL21-(DE3) cells and plated on LB containing ampicillin overnight. All colonies were scrapped into 1L aliquots of terrific broth supplemented with 50 μg/mL ampicillin. Cultures were grown at 37°C and shaken at 220 rpm until an A<sub>600</sub> of 1 was reached, and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 hours at 37°C. Cells were centrifuged at 4000rpm for 18 minutes and stored overnight at 4°C. The bacterial pellet was suspended in 100mM NaCl and 50mM Tris-HCl (pH 7.5) and supplemented with 1 μg/mL leupeptin and 1 mM PMSF. Cells were sonicated for 30 seconds, and rested for 30 seconds, for a total time of 6 minutes. The sonicant was extracted at room temperature for 2 hours in 1g / L bacteria DM, followed by centrifugation at 8000 rpm for 50 minutes. The clarified supernatant was loaded onto a cobalt-affinity column pre-equilibrated with 100mM NaCl, 20mM Tris-HCl and 5mM DM (pH 7.5), and the column was washed until A<sub>280</sub> <0.05. The column was further washed with 40mM imidazole, 100mM NaCl, 20mM Tris-HCl and 5mM DM (pH 7.5), and EC2 was eluted with 400mM imidazole 100mM NaCl, 20mM Tris-HCl and 5mM DM (pH 7.5). Protein was concentrated and purified on a Superdex S-2000 size exclusion column in 100mM NaCl, 10mM HEPES-NaOH, 10mM NaF, 5mM DM (pH 7). Final protein was concentrated to 12-50μM and stored at 4°C.
Fluorescence Anisotropy

200 nM fluorophore labeled monobody in 2mL of 100 mM NaCl, 10 mM NaF, 10 mM HEPES-NaOH, and 5 mM DM (pH 7) underwent emission spectra analysis to ensure adequate fluorophore labeling. 12-50 µM EC2 was added sequentially to the monobody to concentrations of 0.01, 0.03, 0.1, 0.3, and 1 µM. After each EC2 addition the mixture was stirred for 30 seconds and left to incubate for 1 minute. Fluorescence anisotropy was measured after each addition on a Horiba Fluoromax spectrofluorimeter. Each experiment was performed in triplicate. Anisotropy data was fit to a single-site binding isotherm:

\[
A([EC2]) = A_0 + \frac{A_f - A_0}{2} \left(1 + \frac{[EC2]}{[Mb]} + \frac{K_d}{[Mb]} \right) \left(1 - \sqrt{1 - \frac{4[EC2]}{[Mb]} \left(1 + \frac{[EC2]}{[Mb]} + \frac{K_d}{[Mb]} \right)^2} \right)
\]

where \([EC2]\) is the independent variable, \(A_0\) and \(A_f\) are the initial and final fluorescence anisotropy, \([Mb]\) is the monobody concentration, and \(K_d\) is the dissociation constant for the equilibrium reaction:

\[
EC2 * Mb \rightleftharpoons EC2 + Mb
\]

\(K_d\) values for monobody mutants were compared to wildtype via:

\[
\Delta\Delta G^0 = RT\ln \frac{K_{dMut}}{K_{dWT}}
\]

Where \(R = 1.985 \times 10^{-3} \text{ kcal mol}^{-1} \text{ K}^{-1}\). By our analysis, a positive value denoted a weaker binding affinity compared to wildtype.
Results and Discussion

Analysis of the S12-EC2 interface revealed 7 key S12 residues that interact with complementary residues on EC2. S12 residues include T29 on the scaffold, as well as Y78, S80, W84, Y85, E86, and Y87 on the diversified FG loop (Figure 5). T29V and T29S mutations had moderate effects on monobody binding, with T29S showing a greater effect than T29V, though both mutations disrupt possible H-bonding with the carbonyl group of P31 on EC2. Phenylalanine mutations at Y78, Y85, and Y87 showed moderate disruption to binding, each of which is perched within 4.0, 2.6, and 2.7 Å respectively from a H-bond acceptor (Figure 5). Binding could not be measured with the Y78S mutation. Furthermore, mutating Y78 to a tryptophan, reserving aromatic character, led to an indifferent disruption, while mutation to an isoleucine resulted in a moderate change in binding affinity. S80A, with its sidechain only 3.4 Å from the carbonyl group of N27 on EC2 shows a moderate effect on affinity. S12 E86 was identified as the sole possible salt bridge donator, with
the ability to interact with the side chain EC2 K66, which lies 4.7 Å away. Neutralizing the acceptor (E86Q) or moving it farther away from K66 (E86D) both led to moderate effects.

The single biggest difference structurally between S9 and S12 is the insertion of a tryptophan in the FG loop region of S12. Surmising that this change may have important structural implications for the S12-EC2 complex, W84 was mutated to a histidine to preserve aromatic character, a tyrosine to preserve bulkiness and H-bonding character, and a leucine to remove all H-bonding, while keeping the side chain as bulky as naturally possible. W84L and W84H abolished the binding, while W84Y showed moderate defects. Visual comparison of ΔΔG⁰ shown in Figure 6. S12 monobody affinity (Kₐ) and ΔΔG⁰ values reported in Table 1.

![Figure 6: ΔΔG⁰ values for S12 mutants as compared to wildtype.](image)

Orange bars denote “severe” mutations, whose Kd values were beyond the limit of the assay. Blue marks “indifferent”, denoting mutations whose ΔΔG⁰ value falls below 0.6 kcal/mol, thermal energy at room temperature. Green bars are “moderate” mutations, falling between these thresholds.
<table>
<thead>
<tr>
<th>Monobody</th>
<th>$K_d$ (nM)</th>
<th>$\Delta \Delta G^0$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10 ± 6</td>
<td>---</td>
</tr>
<tr>
<td>T29S</td>
<td>620 ± 80</td>
<td>2.43 ± 0.07</td>
</tr>
<tr>
<td>T29V</td>
<td>112 ± 34</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Y78F</td>
<td>88 ± 37</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Y78W</td>
<td>22 ± 5</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>Y78I</td>
<td>180 ± 80</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>Y78S</td>
<td>&gt;3000</td>
<td>&gt;3.4</td>
</tr>
<tr>
<td>S80A</td>
<td>122 ± 15</td>
<td>1.47 ± 0.07</td>
</tr>
<tr>
<td>W84L</td>
<td>&gt;3000</td>
<td>&gt;3.4</td>
</tr>
<tr>
<td>W84Y</td>
<td>628 ± 50</td>
<td>2.45 ± 0.05</td>
</tr>
<tr>
<td>W84H</td>
<td>&gt;3000</td>
<td>&gt;3.4</td>
</tr>
<tr>
<td>Y85F</td>
<td>386 ± 89</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>E86D</td>
<td>265 ± 49</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>E86Q</td>
<td>378 ± 78</td>
<td>2.15 ± 0.04</td>
</tr>
<tr>
<td>Y87F</td>
<td>196 ± 73</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 1: S12 Monobody Mutant affinities.** $K_d$ reported (mean + SEM) from three independent fluorescence anisotropy trials. Positive $\Delta \Delta G^0$ indicates a lower mutant affinity as compared to wildtype S12. Fit anisotropy curves can be found in Supplementary Figure 1S.

Reviewing these results in light of the previous research on S9 at first appears unsurprising. As researchers were unable to increase the binding affinity of the weak binder S9, it would have been even more shocking if we had been successful in increasing the binding affinity of the already tight binder S12, which we were not successful in achieving. Furthermore, a side by side comparison with the analogous mutations in S9 previously reported suggests that S9 was a more
robust monobody, appearing less perturbed by analogous mutations than its S12 counterpart (Figure 7). This is an unsurprising result given the already tight affinity of S12, and further lends to the argument that kcals of energy cannot be measured in angstroms or counted in interactions, as these results show.

![Comparison of ΔΔG^0 values in analogous mutations between S9 and S12 monobody studies.](image)

**Figure 7: Comparison of ΔΔG^0 values in analogous mutations between S9 and S12 monobody studies.** S9 Y84 and S12 Y85 represent analogous residues due to the W84 insertion. *p<0.05. ***p<0.001. ****p<0.0001.

These differences could, furthermore, hint that the 15x difference in affinities between S9 and S12 could be the result of the importance of hydrogen bonding across these residues. S9 and S12 each contain many tyrosine residues that contact the EC2 surface, although not necessarily in the same place. Two of the three tyrosine residues on S9 withstood mutations to phenylalanine and tryptophan without any great change to affinity, suggesting that the aromatic character and volumetric contributions of these residues were enough to keep the S9-EC2 complex stable. On the other hand, S12 tyrosine residues that underwent the smallest change possible, to a phenylalanine, already showed moderate changes in affinity, suggesting that the terminal
hydroxyl groups of these tyrosine residues are more important for S12-EC2 binding than for S9. An interesting residue to note is Y78, found in both S9 and S12, and leading to similar changes in binding affinity when mutated to phenylalanine residues, removing the terminal hydroxyl. A return to normal binding affinity was seen when this tyrosine residue was mutated to a tryptophan in S12, suggesting that volumetric and aromatic contributions may be more important than terminal hydroxyl hydrogen bonding at this residue. It should be noted that the increased frequency of tyrosine residues in S12 and S9 is due to enrichment during the original monobody selection process, but nevertheless these tyrosine residues appear very important partners in this binding complex. It would be premature without further anisotropy and crystallography studies to suggest that these hydrogen bond donors explain the difference in binding affinity, or to pretend to understand the detailed mechanistic and energetic rationale that may explain these differences. What can be inferred is that even in proteins with similar structures, which complete similar functions and show great residue similarity, binding is still too complex a process to attempt to predict the properties of one protein from another.
## Supplemental Figures and Tables

<table>
<thead>
<tr>
<th>S12 Mutation</th>
<th>Forward Primer (5′ -&gt; 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T29S</td>
<td>GATGCTCTCTGAGTTAGGTCATTTTTACGTT</td>
</tr>
<tr>
<td>T29V</td>
<td>GGGATGCTCTCTGAGTTGTCATTTTTACGTTATC</td>
</tr>
<tr>
<td>Y78F</td>
<td>GTATAAGGAAACTTACTACTCCTCTAATCTCTGG</td>
</tr>
<tr>
<td>Y78W</td>
<td>CACTGTATACGCAACTTACTTGGGTCTTCTACTCTCTGGG</td>
</tr>
<tr>
<td>Y78I</td>
<td>CACTGTATACGCAACTTACTACTCCGTCTCTAATCTCTGGG</td>
</tr>
<tr>
<td>Y78S</td>
<td>GTATAAGGAAACTTACTTGGGTCTTCTAATCTCTGGG</td>
</tr>
<tr>
<td>S80A</td>
<td>GCAACTTACTACGCTGCTAATTCTACTCTCTGGTTGCTACG</td>
</tr>
<tr>
<td>W84L</td>
<td>GCTTCTAATCTTGATACGAATACGGGTAGCC</td>
</tr>
<tr>
<td>W84Y</td>
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</tr>
<tr>
<td>W84H</td>
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</tr>
<tr>
<td>Y85F</td>
<td>CTAACACTCTGGTTTGGTTTCAATACGGGTAGCC</td>
</tr>
<tr>
<td>E86D</td>
<td>CTAACACTCTGTGTGGATACGACTACGGTAGCCCAATC</td>
</tr>
<tr>
<td>E86Q</td>
<td>CTAACACTCTGTGTGGATACGACTACGGTAGCCCAATC</td>
</tr>
<tr>
<td>Y87</td>
<td>CTGGTTGGTACGAAATTCCGGTTGAGCCCAATCTCG</td>
</tr>
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</table>

Table S1: Quickchange™ Site Directed Mutagenesis Primers for S12 Mutagenesis. Codons that are in bold and underlines contain the changed base pair(s).
Figure S1: S12 Monobody Binding Isotherms. Fluorescence anisotropy curves fit to Equation (1). Data points reported as mean + SEM. Dotted lines have no theoretical meaning. Reprinted with permission from Turman, D. et al [11] Copyright 2018 American Chemical Society.
References


